REMARKS

Claims 16 and 18-32 and 38-41 are active in this application. The claims have been amended for clarity. No new matter is added

Applicants wish to thank Examiner Mertz for the helpful comments in the Office Action to address the rejections.

The essential inquiry pertaining to the requirement under 35 U.S.C. § 112, second paragraph is whether the claims set out and circumscribe a particular subject matter with a reasonable degree of clarity and particularity. Definiteness of claim language must be analyzed, not in a vacuum, but in light of:

(A) The content of the particular application disclosure;
(B) The teachings of the prior art; and
(C) The claim interpretation that would be given by one possessing the ordinary level of skill in the pertinent art at the time the invention was made. See MPEP § 2173.02

To the phrase "which exhibits IL-2R β-chain binding activity and/or lymphocyte stimulatory activity" this phrase is definite for the following reasons.

The specification describes that there are certain peptides, i.e., those which show at least 75% identity to SEQ ID NO:6 that exhibit specific binding to IL-2R β-chain and not "any peptide" as asserted by the Examiner. The specific binding to IL-2R β-chain is clearly described in the specification, for example, Applicants direct the Examiner's attention to Examples 7 and 9.

In Example 7, the publication Moreau et al (*Mol. Immunol.*, 1995, 32, 1415, 1047-1056) is cited. A copy of this publication is attached for reference. This publication clearly shows what the specific binding to the IL-2R β chain (see page 1049, left column of Moreau). This is also described in Example 9 of the present application.

As one basis for this rejection, the Examiner has taken the position that "it is expected that the binding of the peptide to the IL-2R β-chain would result in stimulation of

lymphocytes." However, it appears that the Examiner has misunderstood the relationship between binding activity and proliferation activity as they can be separate or the proliferation activity may be also obtained after binding, which is described in the specification.

Applicants again refer the Examiner's attention to the Moreau publication, which on pages 1050 and 1051, col. 1 states:

> mAB 19B11/β specifically inhibits the binding of human IL-2 to human IL-2Rβ while 16F11/α inhibits the binding to human IL-2R α. . .
>
> the proliferation of TS1β was very sensitive to the effect of mAB 19B11/β (figure 3). This inhibition is probably related to the inhibition of IL-2 binding. However, at 100-fold higher concentrations, mAB 16F11/α and 3H9 also affect the proliferation of TS1β. This effect may be related to an inhibition of internalization of the complexes formed by IL-2 and IgG molecules." (emphasis added).

In view of the above, the specification clearly describes that the specific binding activity and the proliferation activity (e.g., CD3, CD8, and NK cells--see page 19 of the present specification) can be dissociated. The fact that the two activities can be dissociated provides on one hand the ability to detect IL-2R in a cell, for example, as described on page 32 and 33 and on the other hand may also stimulate lymphocytes.

The binding activity apart from the lymphocyte stimulatory activity is also described in the attached Morris et al publication (*Ann. Rheum. Dis.*, 2000, 59(SI), i109-i114). Morris et al describes peptide binding to IL2rα which in turn can be used to treat graft versus host disease (see page i111, 2nd column).

Thus, the phrase "which exhibits IL-2R β-chain binding activity and/or lymphocyte stimulatory activity" is definite in view of the present specification, the knowledge in the art; and based on the claim interpretation that would be given by one in the art.

The remaining issues raised by the Examiner have been addressed by amendment.

In view of the above, withdrawal of the rejection under 35 U.S.C. § 112, second paragraph is requested.

Applicants request that this case be passed to issuance.

Respectfully submitted,

OBLON, SPIVAK, McCLELLAND,
MAIER & NEUSTADT, P.C.

Norman F. Oblon
Attorney of Record
Registration No. 24,618

Customer Number
22850

Daniel J. Pereira, Ph.D.
Registration No. 45,518

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CHARACTERIZATION OF A MONOCLONAL ANTIBODY DIRECTED AGAINST THE NH2 TERMINAL AREA OF INTERLEUKIN-2 (IL-2) AND INHIBITING SPECIFICALLY THE BINDING OF IL-2 TO IL-2 RECEPTOR β CHAIN (IL-2Rβ)

JEAN-LOUIS MOREAU,* MARC BOSSUS,† DONAT DE GROOTE,‡ CHRISTINE FRANÇOIS,§ YANNICK JACQUES,§ ANDRÉ TARTAR† and JACQUES THÈZE*¶

*Unité d'Immunogénétique Cellulaire, Institut Pasteur, 25 rue du Dr Roux, 75015 Paris, France;
†Unité de Chimie des Biomolécules -URA CNRS 1309, Institut Pasteur de Lille, 20 Boulevard Louis XIV, 59000 Lille, France;
‡Medgenix, B-6220 Fleurus, Belgium;
§Unité INSERM U211, Institut de Biologie, 9 Quai Moncousu, 44035 Nantes Cedex, France

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Abstract—An anti-human IL-2 mAb (19B11/β) was found to selectively block the binding of IL-2 to TS1β cells expressing the interleukin-2 receptor β (IL-2Rβ) without affecting binding to TS1α cells expressing the IL-2Rα receptor. It also specifically inhibits the IL-2 driven cell proliferation in TS1β cells. These observations have lead to the hypothesis that its epitope is related to an IL-2 area involved in binding with IL-2Rβ chain. This epitope was identified using various peptides covering the N-terminal half (including α helix A) of the 133 amino acids of IL-2. MAb 19B11/β does not recognize peptides 30–54 and 44–54 but recognizes peptides 1–22 and 1–30 with a good affinity. Furthermore, threonine in position no. 3 was found to be critical for the binding of mAb 19B11/β. A relationship between the epitope of mAb 19B11/β and the glycosylation of the IL-2 molecule was observed. This further demonstrates that the NH_2 terminal area of IL-2 is critical for IL-2/IL-2Rβ interactions. Two other mAbs were studied during the course of this work. They served as control for the study of mAb 19B11 β and provide some additional insight concerning the question of IL-2/IL-2R structure–function. MAb 16F11 α selectively blocks the IL-2 binding to TS1α cells. The epitope of mAb 16F11 is conformational and it was not possible to study the corresponding IL-2/IL-2Rα region of interaction. Epitope of mAb 3H9 is localized between residues 30 and 54 and does not affect the binding of IL-2 to IL-2Rα.

Key words: IL-2, IL-2 receptor, anti-IL-2 mAb.

INTRODUCTION

Interleukin-2 (IL-2) is a 133 aa polypeptide of 15–18 kDa depending on e degree of glycosylation (Bazan, 1992; Robb *et al.*, 1984; Smith, 1988; Taniguchi *et al.*, 1983). It is secreted by activated T cells and despite the increasing number of cytokines able to promote T cell proliferation (IL-4, IL-7, IL-9, IL-6, TNFα etc.), IL-2 remains the main T cell growth factor. IL-2 also modulates the activation, proliferation and differentiation of other cells in the immune system including NK-cells, B cells and monocytes (Smith, 1988).

The effects of IL-2 on these various cells are mediated through specific cell surface receptors (IL-2R) (Minami *et al.*, 1993; Taniguchi and Minami, 1993). Over the past few years, our understanding of the IL-2R complex has increased substantially. It is now known that the IL-2R comprises at least three subunits encoded by different genes. These subunits can be expressed individually or in various combinations resulting in receptors that bind IL-2 with markedly different affinity. The first IL-2R component to be identified, IL-2Rα, is a 55-kDa protein that binds IL-2 with a K_d of ~10 nM (Leonard *et al.*, 1984; Uchiyama *et al.*, 1981). The immunochemistry of IL-2Rα (Kumar *et al.*, 1987; Malek *et al.*, 1983; Moreau *et al.*, 1987) and the role of IL-2 on IL-2Rα gene expression have been studied (Froussard *et al.*, 1991; Jankovic *et al.*, 1989). The second IL-2R component, IL-2Rβ, is a 75-kDa protein with a large intracytoplasmic domain (286 aa) that plays an important role in receptor-mediated signaling (Hatakeyama *et al.*, 1989; Teshigawara *et al.*, 1987; Tsudo *et al.*, 1989). The third component recently identified, IL-2Rγ is a 64-kDa protein (Ishii *et al.*, 1994; Takeshita *et al.*, 1992). IL-2Rβ and IL-2Rγ alone have a very low affinity for IL-2. However, when expressed

¶Author to whom correspondence should be addressed.
Abbreviations: aa, amino acid; IL-2, interleukin-2; IL-2R, interleukin-2 receptor; K_d, dissociation constant; mAb, monoclonal antibody.

together, these two chains form an intermediate affinity receptor with a K_d of ~1 nM. Expression of the three receptors is required to form a high affinity IL-2R (K_d ~10 pM). IL-2Rα does not exhibit homology with the cytokine receptor family while IL2Rβ and -γ belong to that family. The IL-2Rβ chain is also part of the IL-15R complex. The IL-2Rγ is common to IL-4R, IL-7R, IL-9R and IL-15R (Thèze, 1994). Heterodimerization of IL-2Rβ and IL-2Rγ is required for signal transduction. The dimer binds tyrosine kinase JAK1 and JAK3 and this represents the primary event during the IL-2-mediated T cell activation (Nakamura *et al.*, 1994).

Numerous studies employing mutagenesis have been performed to probe IL-2/IL-2 receptors interactions. In the mouse system this has led to the identification of two critical sites for IL-2 binding to IL-2Rβ and IL-2Rγ. While aspartic acid in position 34 is essential for the binding to IL-2Rβ, glutamine in position 141 is involved in binding to IL-2Rγ. Nineteen amino acids are important for IL-2 binding to IL-2Rα (Zurawski *et al.*, 1993; Zurawski and Zurawski, 1992). Much less is known about human IL-2 receptor binding. In one study a single mutation near the N-terminus of IL-2 (Asp 20) was reported to specifically perturb the interaction between IL-2 and IL-2R of intermediate affinity (Collins *et al.*, 1988; Flemming *et al.*, 1993; Ju *et al.*, 1987).

We have previously characterized a family of eight monoclonal antibodies (mAbs) able to affect differentially the binding of human IL-2 to either the α chain or the $\beta\gamma$ complex (Rebollo *et al.*, 1992). In the present study we have analysed in more detail the properties of mAb 19B11/β able to specifically inhibit the binding of IL-2 to IL-2Rβ. We also report on the properties of mAb 16F11/α —inhibiting the binding of IL-2 to IL-2Rα — and some characteristics of mAb 3H9 that do not affect the binding of IL-2 to any of the IL-2Rs. These results extend our previous results and demonstrate that, in agreement with data from mutagenesis experiments, the NH_2 terminal part of human IL-2 is critical for binding to IL-2Rβ.

MATERIALS AND METHODS

Reagents and antibodies

Human non-glycosylated rIL-2 was a generous gift of Roussel Uclaf (Romainville, France). Human glycosylated rIL-2 produced in Chinese hamster ovary (CHO) cells was kindly provided by Dr Ferrara (Sanofi, Castanet-Tolosan, France).

Supernatant from Hela subline (H28) transfected with the plasmid pKCRIL-4 was kindly provided by Dr T. Honjo (Kyoto University, Japan) and was used as a source of murine rIL-4 (Severinson *et al.*, 1987).

The soluble β chain has been produced from RNAs extracted from YT-2C2 cells. After extraction, cDNAs has been obtained by treatment with a DNA polymerase. Two oligonucleotides, one of them specific for the domain near the initiation site, the other one specific for the sequence near the sequence coding for transmembrane domain, have been constructed and used to amplify the cDNA fragment coding for the extracellular domain of the β chain. Then, this fragment was inserted into an expression vector, pKCR6 (Matrisian *et al.*, 1986), to realise a stable transfection into CHO cells. Secreted product was purified using an immunoaffinity column with a monoclonal anti-β chain (CF1).

Mouse monoclonal antibodies 19B11/β (IgG1), 16F11/α (IgG2a) and 3H9 (IgG1) were produced as previously described (Rebollo *et al.*, 1992). Briefly, female BALB/c mice were repeatedly immunized with 5 μg of human non-glycosylated rIL-2. Splenocytes were fused with NSØ myeloma cell line. After fusion, screening for anti-IL-2 activity was carried out by immunoprecipitation in liquid phase. After cloning, the reactivity of anti-IL-2 mAbs was tested by ELISA. Isotypes of anti-11-2 mAb were determined using the kit supplied by Miles Scientific (Naperville, IL). Rat monoclonal 11B11 (IgG, k), specific for murine IL-4, was provided by Dr W. Paul (National Institute of Health, Bethesda, MD). Goat anti-mouse polyvalent immunoglobulin alkaline phosphatase conjugate was from Sigma (St. Louis, MO).

Mouse polyclonal anti-human IL-2 antibodies were prepared in the laboratory. BALB/c animals were immunized by repeated injection of 50 μg of IL-2 in CFA and were regularly bled. A pool from 10 animals was used for purification.

MAbs and polyclonal antibodies were purified in two steps: ammoniac sulfate precipitation was followed by chromatofocusing as described by the supplier (Pharmacia, France).

Cell lines and culture media

TS1 cells were maintained in complete medium supplemented with supernatant of recombinant baculovirus expressing murine IL-9 proteins (DIB 349) (Uyttenhove *et al.*, 1988).

TS1β cells were obtained after transfection of TS1 cells with human p70 IL-2R CDNA cloned in the pdKCR expression vector kindly provided by Dr T. Taniguchi (Institute for Molecular and Cellular Biology, Osaka University, Osaka, Japan). TS1$\alpha\beta$ cells were obtained after transfection of TS1β cells with human p55 IL-2R cDNA cloned in pCMV4 expression vector kindly given by Drs W.A. Kuziel and W.C. Greene (Gladstone Institute Virol. Immunol., San Francisco, CA). TS1β and TS1$\alpha\beta$ were previously characterized (Pitton *et al.*, 1993) and TS1β and TS1$\alpha\beta$ cell lines were grown as TS1 cells in complete medium supplemented with IL-9.

YT cells were generous gift from Dr. J. Yodoï (Kyoto University, Kyoto, Japan) and were maintained in complete medium (Yodoi *et al.*, 1985).

All cultures were performed in complete medium composed of RPMI 1640 (BioProducts, Walkerville, MD), 10% heat inactivated FCS (Serovial, Vogelgsun, France), 2 mM glutamine, 100 units ml penicillin, 100 μg ml streptomycin and 50 mM 2-β-mercaptoethanol (2β-ME).

Proliferation assay

Cells were cultured (10^4 cells/well) in 96-wells plat-bottomed microtiter plates in a final volume of 0.2 ml. Different concentrations of human rIL-2 and mouse rIL-4 were tested.

Concentrations giving about half maximal proliferation were used for inhibition experiments. The same concentrations were used for TS1αβ and TS1β (rIL-2, ng/ml; IL-4, 12.5 U/ml). Serial dilution of anti-IL-2 or anti-IL4 mAb antibodies were mixed in the culture wells with the respective lymphokines 30 min before adding the cells.

Cultures were pulsed with 0.5 μCi/well of (^{3}H) TdR after 36 hr of incubation and harvested 15 hr later.

IL-2 binding assay and inhibition

The IL-2 binding assay was performed as follows. Serial dilutions of ^{125}I-labelled IL-2 were incubated with 5×10^5 cells in RPMI 1640 medium containing 10 mg/ml BSA, 25 mM Hepes and 0.2% sodium azide in a total volume of 100 μl for 30 min at 37°C. After incubation, cell-bound radioactivity was separated from free radioactivity by centrifuging the cells through a 200 μl layer of a mixture of 84% silicone oil (DC 550, Serva, Germany) and 16% paraffin oil (Neyol Laboratories, Fimouze, France) in 400 μl polyethylene tubes. The bottoms of the tubes containing the cell pellets were cut off and the radioactivity was measured.

For inhibition experiments, 2.5 ng ^{125}I-IL-2 were used per well. Serial dilution of mAbs were preincubated with ^{125}I-IL-2 during 30 min before adding the cells. The same protocol was used to study the inhibitory capacity of soluble IL-2Rβ

ELISA, binding inhibition and affinity measurements

ELISA was used to test the reactivity of anti-IL-2 mAb against rIL-2 and peptides. Wells were coated with a solution containing 1 μg/ml of IL-2 or peptides in K_2HPO_4/KH_2PO_4, 0.05 M, pH 8 buffer. After overnight incubation at 4°C, wells were washed and saturated with bovine serum albumin (BSA). Several dilutions of purified anti-IL-2 mAb (or purified polyclonal antibodies) were added for 1 hr at 37°C. Wells were washed (PBS–Tween 20, 0.1%) followed by the addition of an alkaline phosphatase anti-mouse Ig conjugate. After 1 hr at 37°C wells were washed and substrate (Sigma 104 phosphatase substrate) was added at 1 mg/ml. The O.D. (405 nm) was read in a spectrophotometer after 1 hr at 37°C.

For the inhibition experiment, a concentration of mAbs giving half maximal binding was used (19B11/β, 0.1 μg/ml; 16F11/α; 0.4 μg/ml; 3H9, 0.1 μg/ml). This solution was mixed during 1 hr at 37°C with different concentrations of inhibitor before addition to the wells.

Affinities (K_d) were calculated according to Friguet *et al.* (1985). In some experiments. IL-2 was treated by 2β-ME. IL-2 (20 μg/ml) diluted in K_2HPO_4/KH_2PO_4, 0.05 M, pH 8 buffer was mixed with different concentrations (from 0.04 M to 5 M) of 2β-ME during 1 hr at room temperature. This mixture (50 μl/wells) was directly used for coating the cells used for ELISA as described above.

Peptide synthesis

Peptides were synthesized by stepwise solid-phase method according to Boc/trifluoroacetic acid scheme (Merrifield, 1963), on a *p*-methylbenzhydrylamine resin (Applied Biosystems) in an Applied Biosystems 430A peptide synthesizer. Tri-functional amino acids were protected as follows: Arg (tosyl), Asp (*O*-cyclohexyl ester), Glu (*O*-cyclohexyl ester), Lys (Nε-2-chlorobenzyl-carbonyl), Ser (benzyl ether), Thr (benzyl ether), His (dinitrophenyl), Met (sulfoxyde), Arg (tosyl), Tyr (2-bromo-benzyl). At the end of the synthesis the Boc group was removed and the N-terminal function was acetylated with acetic anhydride. Final deprotection and cleavage of the peptidyl resin was performed by treatment with hydrogen fluoride for 1 hr at 0°C in the presence of *p*-cresol. For the peptides containing a methionine, this operation was preceded by treatment with HF DMS to remove the sulfoxyde group. The cleaved deprotected peptides were precipitated with cold diethylether, dissolved in 5% acetic acid and lyophilized.

Crude peptides were purified by reverse-phase preparative HPLC. After purification, peptides were checked for identity by mass spectrometry and amino acid analysis after total hydrolysis.

Pepscan

The overlapping 10-mer peptides spanning the 133 amino acids of the IL-2 sequence were prepared using Pepscan on polyethylene rods according to the procedure described by Geysen *et al.* (1984). At the end of the synthesis, the amino-terminal protecting group was removed and the peptides were acetylated using acetic anhydride.

Binding of the mAb to the different decamers was performed as described in the ELISA section.

RESULTS

Inhibition of binding of radiolabelled IL-2 by mAbs

To assay the effects of mAb 19B11/β on IL-2 binding, two cell types were used. These two cell clones (TS1α and TS1β) were isolated from the same mouse T cell (TS1) after transfection either by human IL-2Rα gene or IL-2Rβ gene. Since TS1 cells spontaneously express only mouse IL-2Rγ (γ_{mu}) they do not bind radiolabelled IL-2. Therefore, the IL-2 binding observed with TS1α and TS1β is strictly dependent on the presence of human IL-2R α_{hu} or IL-2Rβ_{hu}.

MAb 19B11/β specifically inhibits the IL-2 binding to TS1β while mAb 16F11/α or 3H9 have no effect on this binding (Fig. 1). However, even at high concentrations of 19B11/β, the inhibition was not complete. On TS1α cells only mAb 16F11/α inhibit completely the binding. Surprisingly, with TS1α cells mAb 19B11/β and 3H9 enhance the IL-2 binding in a narrow range of con-

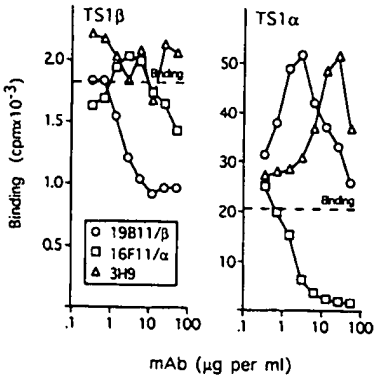

Fig. 1. Inhibition of IL-2 binding by mAb. The IL-2 binding assay was performed as described in Materials and Methods using TS1α cells (IL-2R$\alpha_{hu/mu}$) or TS1β cells (IL-2R$\beta_{hu/mu}$). The effect of serial dilutions of mAbs 19B11/β (-○-○-), 16F11/α (-□-□) and 3H9 (-△-△-) was assayed on the binding of 2.5 ng of ^{125}I-IL-2 to 5 × 10^5 cells. The binding obtained without inhibitor (——Binding) was 1800 cpm and 21 000 cpm for TS1β and TS1α cells, respectively. The results obtained in a representative experiment are shown.

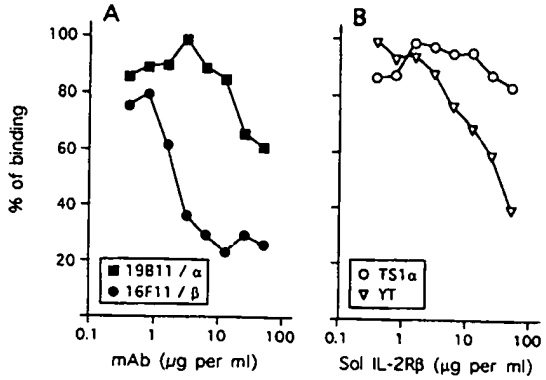

Fig. 2. Inhibition of IL-2 binding by mAb and soluble IL-2Rβ. The IL-2 binding assay was performed as described in Materials and Methods and using TS1α cells (IL-2R$\alpha_{hu/mu}$) or YT cells (IL-2R$\beta_{hu/mu}$). (A) The effect of serial dilution of mAbs 19B11/β (-●-●-) and 16F11/α (-■-■-) was assayed on the binding of 2.5 ng of ^{125}I-IL-2 to 5 × 10^5 YT cells. (B) The effect of different concentrations of soluble IL-2Rβ was assayed on the binding of 2.5 ng of ^{125}I-IL-2 to 5 × 10^5 TS1α cells (-○-○-) or YT (-▽-▽-). The results obtained with the two cell lines and the two inhibitors are expressed as % of binding without inhibitor.

centrations (Fig. 1). This effect could be attributed to a conformational change induced on IL-2 by mAb 16F11/α and 3H9 that would enhance binding to the hybrid $\alpha_{hu/mu}$ IL-2α. Alternative explanations are discussed below.

One can conclude that mAb 19B11/β specifically inhibits the binding of human IL-2 to human IL-2Rβ while mAb 16F11/α inhibits the binding to human IL-2Rα. This is in keeping with our previous observations (Rebollo *et al.*, 1992).

Inhibition of binding of radiolabelled IL-2 by soluble IL-2Rβ

The inhibitory capacity of mAb 19B11/β was verified on human YT cells which express spontaneously the human β and γ IL-2R but not the α receptors and thus resemble to TS1β but the γ receptor is from human origin. In these cells, the inhibitory capacity of mAb 19B11/β reaches 80% under the experimental conditions used. Under the same experimental conditions, mAb 16F11/α does not inhibit significantly the binding (Fig. 2).

Using YT cells we have compared the effects of mAb 19B11/β with the effects of soluble IL-2Rβ. As expected, soluble IL-2Rβ does not inhibit the binding of radiolabelled IL-2 to TS1α cells but does partially inhibit the binding of radiolabelled IL-2 to YT cells (Fig. 2).

MAb 19B11/β has inhibitory properties comparable to IL-2Rβ and therefore may bind to a similar region of the IL-2 molecule.

Neutralization of IL-2 proliferation by mAb 19B11/β

TS1β cell line was shown to proliferate in a dose-dependent manner in response to IL-2. This response is strictly independent on the expression of IL2Rα receptor (Pitton *et al.*, 1993). This cell also proliferates in response to IL-4 or IL-9. TS1αβ cell line was obtained after transfection of TS1β with human IL2Rα gene. This cell line is dependent on the expression of IL-2Rα for IL-2 dependent cell proliferation (Pitton *et al.*, 1993). Using these cell lines the effects of mAb 19B11/β and 16F11/α and 3H9 were assayed on the IL-2 dependent proliferation.

The proliferation of TS1β was very sensitive to the effect of mAb 19B11/β (Fig. 3). This inhibition is probably related to the inhibition of IL-2 binding. However,

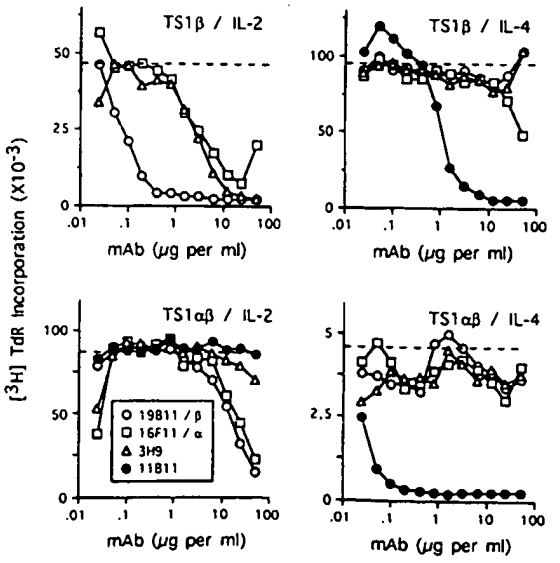

Fig. 3. Inhibition of IL-2 proliferation of TS1β and TS1αβ. The proliferation assay was conducted as described in Materials and Methods using 10^4 cells well in a final volume of 0.2 ml. For the inhibition experiment 1 ng ml of human rIL-2 or 12.5 U ml mouse rIL-4 were used. The proliferation of TS1β or TS1αβ obtained with these doses of lymphokines is indicated by the dashed line. For each cell line the effects of different concentrations of mAbs is shown: -○-○-19B11 β: -□-□-16F11/α: -△-△-3H9 and - - -11B11. The result of a representative experiment is shown.

at 100-fold higher concentrations. mAb 16F11/α and 3H9 also affect the proliferation of TS1β. This effect may be related to an inhibition of internalization of the complexes formed by IL-2 and IgG molecules. The specificity of these effects is shown by the fact that the three mAbs have no significant effects on the IL-4 proliferation of TS1β under conditions in which mAb 11B11 (anti-mouse IL-4) gives a complete inhibition (Fig. 3).

The proliferation of TS1αβ is inhibited by mAb 19B11/β as well as by mAb 16F11/α thus confirming that this line is dependent on the expression of high affinity IL-2R for proliferation and that mAb 19B11 which affect the binding to IL-2Rβ also affects the biological response. As with TS1β cell line, the IL-4 proliferation of TS1αβ is not affected by the three anti-IL-2 mAbs (Fig. 3).

One may notice that 0.05 μg/ml of mAb 19B11/β is necessary for 50% inhibition of the proliferation of TS1β while 10 μg/ml is necessary for the same inhibition of TS1αβ. These 200-fold differences are explained by the difference in affinity between the βγ IL-2R (10^{-9} M) and the αβγIL−2R (10^{-11}–10^{-12} M).

Effects of disulfide bond reduction on IL-2 recognition by mAbs

The presence of the disulfide bound between cysteine 58 and cysteine 105 has been shown to be critical for IL-2 structure and biological activity. The effect of 2-β-mercaptoethanol (2β-ME) on the conformation of the epitopes recognized by mAb 19B11/β, 16β/α and 3H9 was assayed. Mouse polyclonal antibodies made against human IL-2 were also used in these experiments.

Under the experimental conditions described in Materials and Methods, treatment with 2β-ME affects neither the binding of mAb 19B11/β nor the recognition of IL-2 by polyclonal antibodies or mAb antibody 3H9. Under the same conditions, the binding of mAb 16F11/α is greatly affected by 2β-ME treatment (Fig. 4).

This preliminary experiment suggests that the epitope recognition by mAb 16F11/α is highly conformational and is not likely to be mimicked by linear peptides. In contrast, the results obtained with mAb 1911/β suggest that it is possible to pursue the characterization of a linear peptide involved in its epitope.

Localization of the epitope of mAb 19B11 β

Four peptides were synthetized to assay the specificity of mAb 19B11/β. Peptides 1–22 and 1–30 cover the NH_2 terminal tail of IL-2 and either partially (1–22) or totally (1–30) the first α helix (α helix A). Peptides 30–54 covers all the loop joining α helix A to α helix B-B'. Peptide 44–54 covers an area including peptide 44–46 which may fold in a β-sheet fashion.

Figure 5 shows that mAb 19B11 β recognize very specifically peptides 1–22 and 1–30. No signal was obtained in ELISA on peptides 30–54 and 44–54. As expected, mAb 16F11/α does not bind to any of these peptides even though it binds to IL-2.

In the same assay polyclonal antibodies bind strongly to peptides 1–30 thus suggesting that this part of IL-2 if

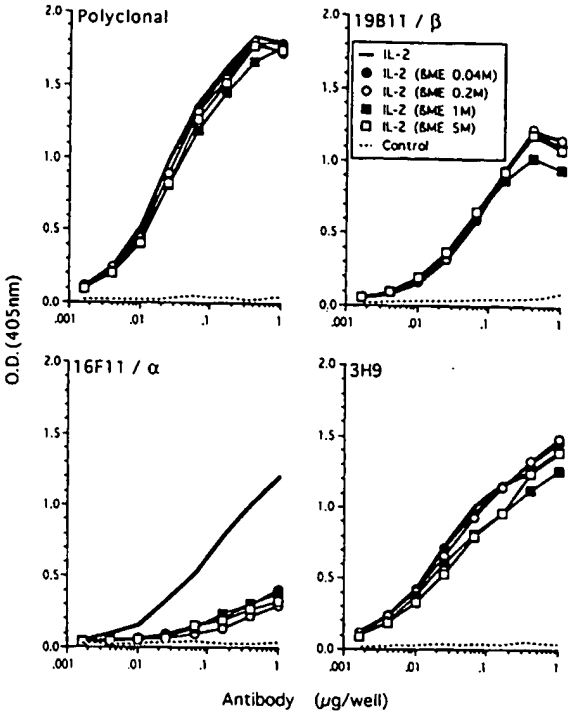

Fig. 4. Effect of β-mercaptoethanol treatment of IL-2 on the binding of polyclonal and mAbs. Before coating the plates a solution of IL-2 (20 μg/ml) was treated by different concentrations of β-ME (-●-●-: 0.04 M, -○-○: 0.2 M, -■-■-: 1 M, -□-□-5 M) during 1 hr at room temperature. Fifty microliters of these solutions were used to coat the plates. The ELISA technique used to detect the binding of purified polyclonal and mAb antibodies to β-ME-treated IL-2 is described in Materials and Methods. The results are directly given as the O.D. obtained after 1 hr incubation at 37°C.

very immunogenic. They bind less efficiently to 1–22 and 30–54 while the binding to 44–54 is very weak. Contrary to mAb 19B11/β, mAb 3H9 binds to peptide 30–54 thus showing the specificity of the system. Surprisingly, mAb 3H9 binds peptide 1–22 but more weakly peptide 1–30 although it completely encompasses the 1–22 sequence (Fig. 5). Inhibition experiments have however confirmed the full specificity of the system (see below).

Peptide inhibition of IL-2 binding and affinity measurements

Different concentrations of peptides were used to inhibit the IL-2 binding by mAbs 19B11/β, 16F11/α and 3H9 using the technique described in Materials and Methods (Fig. 6).

The binding of 19B11/β was inhibited only by peptides 1–22 and 1–30, thus confirming the binding results. The binding of 3H9 was efficiently inhibited only by peptides 30–54 and 44–54. These results confirm the complete specificity of the system. As expected, IL-2 binding by 16F11/α was not affected by these peptides.

From these data the affinities for the different peptides were calculated (see Materials and Methods and Table 1). MAb 19B11 β has a good affinity for IL-2 (K_d 2 ×

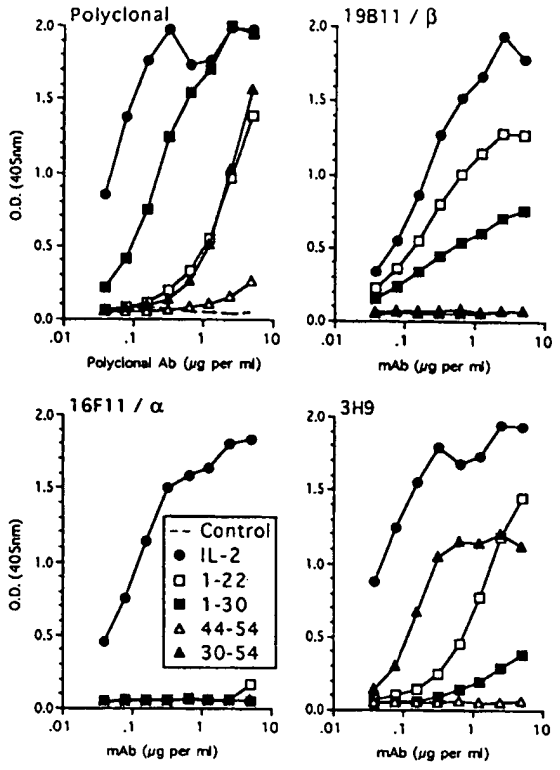

Fig. 5. Binding of mAbs to IL-2 peptides. The ELISA plates were first coated with IL-2 (-●-●-), peptide 1–22 (-□-□-), peptide 1–30 (-■-■-), peptide 44–54 (-△-△-) or peptide 30–54 (-▽-▽-) using a solution at 1 μg/ml. The ELISA technique used thereafter is described in Materials and Methods. The results obtained with the purified polyclonal antibodies and purified mAbs 19B11/β, 16F11/α and 3H9 are expressed as the O.D. obtained after 1 hr incubation at 37°C.

10^{-9} M) and peptide 1–30 (K_d 5 × 10^{-7} M). Surprisingly, mAb 3H9 has a stronger affinity for peptide 30–54 (K_d 1.3 × 10^{-9} M) than for IL-2 (K_d 1.5 × 10^{-8} M). This situation where the antibodies have a stronger affinity for a molecule not used as an immunogen has already been observed (heteroclicity).

Importance of threonine in position 3 in the epitope of mAb 19B/11β

The peptide which shows the strongest affinity for mAb 19B11/β corresponds in the folded IL-2 to a tail of 10 aa and an α helix of 20 aa. Using the pepscan method described in Materials and Methods and a series of decapeptides, we have evaluated the role of each aa in binding of 19B11/β to peptide 1–30. Figure 7 shows these results. As controls, mAbs 16F11/α and 3H9 do not bind to the series of decapeptide examined here. In contrast, mAb 19B11 binds very strongly to peptides 1–10. It also binds to peptides 2–11 and 3–12 but the loss of threonine 3 in peptide 4–13 leads to the disappearance of decapeptide recognition and no binding is observed.

Since threonine 3 is known to be the unique site of IL-2 glycosylation, we have compared the recognition of glycosylated IL-2 and non-glycosylated IL-2 by mAb 19B11/β. In ELISA, mAbs 16F11/α and 3H9 recognized these two molecules in a comparable manner. In contrast, the pattern of recognition by mAb 19B11/β is dramaticly different; this mAb does not recognize glycosylated IL-2. This is in agreement with the fact that non-glycosylated IL-2 was used for the production of mAb 19B11/β (Fig. 8).

DISCUSSION

Considering the important immunoregulatory role of IL-2, and the therapeutic promise it holds for the treatment of certain cancers and infection diseases, this cytokine has been the focus of several structure–function studies aimed at characterizing agonists, antagonists of the molecules.

A major step towards this goal has been given by the determination of the three-dimensional structure of IL-2 (Bazan, 1992). IL-2 as several other cytokines, including human growth hormone and prolactin, and these have structures composed of a compact core bundle of four antiparallel α helices. For human IL-2, helix A extends from aa 11 to 27, helix B-B′ from aa 55 to 74, helix C from aa 84 to 97 and helix D from aa 115 to 133. These helices are connected by three loops: A-B (aa 28–54); B-C (aa 75–83) and C-D (aa 98–114). Therefore, Asp 20 which is critical for binding to the IL-2R of intermediate affinity is localized in the middle of α helix A. In a more recent and detailed study the role of the segment 17–21 (Leu-Leu-Leu-Asp-Leu) surrounding Asp 20 has been investigated. It was found that Asp 20 and Leu 21 are the functionally most important residues in this region. Asp 20 is solvent accessible and likely plays a direct receptor contact role. Leu 21, in contrast, is completely buried in the hydrophobic area of the protein and substitutions at this position perturbs the hydrophobic packing arrangements important for biological activity (Berndt *et al.*, 1994).

Several lines of evidence demonstrate that mAb 19B11/β specifically inhibits the binding of human rIL-2 to IL-2Rβ.

(1) Using the same mouse T cell (TS1) —expressing only mouse γ chain — transfected either with human IL-2Rα or human IL-2Rβ, it has been possible to obtain cell lines which bind IL-2 through either the receptor IL-2Rγ_{mu}/IL-2Rα_{hu} (TS1α) or the receptor IL-2Rγ_{mu}/IL-2Rβ_{hu} (TS1β). Under the experimental conditions discussed in this paper, only mAb 19B11/β inhibit the binding to TS1β.

(2) This has been further documented by showing that mAb 19B11/β and soluble IL-2Rβ inhibit the IL-2 binding to human YT cells expressing the human IL-2Rβγ. Neither of these two molecules. however, give a complete inhibition of the binding and the respective amounts required for half maximal inhibition is compatible with the fact that soluble IL-2Rβ exhibits a low affinity for IL-2 (K_d 10^{-7} M).

(3) Functionally 19B11 β inhibits the growth effects of IL-2-mediated either by the intermediate affinity receptor complex (βγ) or by the high affinity receptor complex

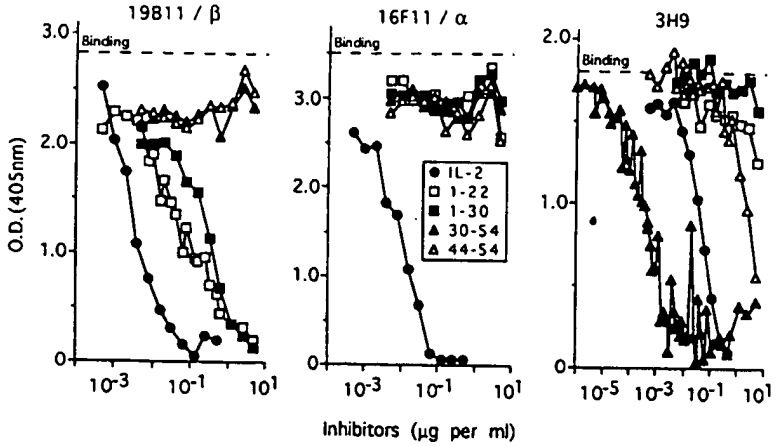

Fig. 6. Inhibition of IL-2 binding to mAbs by IL-2 peptides. These experiments were performed using the ELISA technique described in Materials and Methods. The plates were coated with rIL-2 (1 μg/ml). Concentration of mAb antibodies giving half maximal binding were used (19B11/β and 3H9 at 0.1 μg/ml; 16F11 α at 0.4 μg/ml). The corresponding binding is shown in each panel of the figure (see dashed line). The antibodies were mixed with various concentrations of these different peptides before addition to the plates: IL-2 (-●-●-), peptide 1–22 (-□-□-), peptide 1–30 (-■-■-), peptide 30–54 (-▲-▲-) and peptide 44–54 (-△-△-). The affinities of IL-2 and of the different peptides were calculated from this data (see Results and Table 1).

Table 1. Affinity of the mAb for IL-2 or IL-2 peptides (Kd in M)[a]

	IL-2	1–22	1–30	30–54	44–54
19B11/β	2.1×10^{-9}	1.3×10^{-7}	4.9×10^{-7}	$>10^{-5}$	$>10^{-5}$
16F11/α	3.2×10^{-9}	$>10^{-5}$	$>10^{-5}$	$>10^{-5}$	$>10^{-5}$
3H9	1.4×10^{-6}	4.5×10^{-6}	$>10^{-5}$	1.3×10^{-9}	5.3×10^{-6}

[a] Affinities were calculated from the data represented in Fig. 7 using the method described by Friguel *et al.* (35).

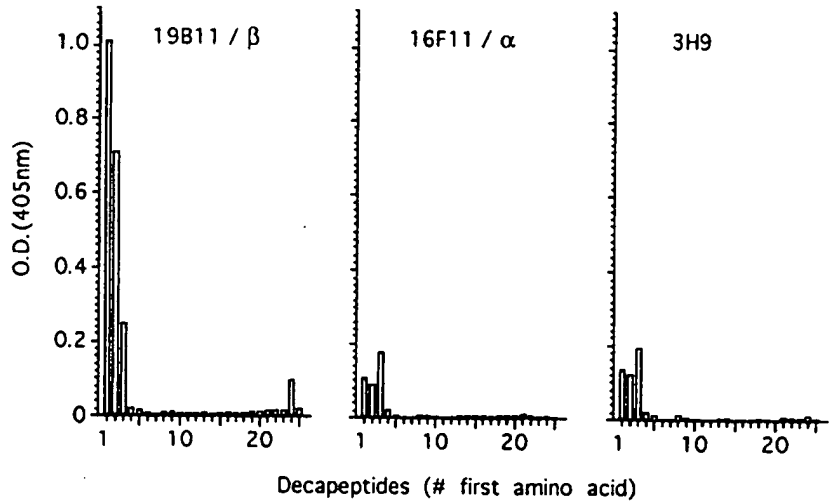

Fig. 7. Pepscan analysis of mAbs binding. The overlapping 10-mer peptides spanning the 133 amino acids of the IL-2 sequence were prepared using Pepscan on polyethylene rod as described in Materials and Methods. The binding of the mAb antibodies was then assayed using the ELISA technique described in Materials and Methods. The data are represented only for the first 25 decamers.

($\alpha\beta\gamma$). The pattern of inhibition and the dose of 19B11/β required is compatible with a blockade of the interaction between IL-2 and the IL-2Rβ chain.

(4) All these effects have been shown to be specific since mAb 16F11/α, which specifically affects the binding of radiolabelled IL-2 to rIL-2α, has been used as control.

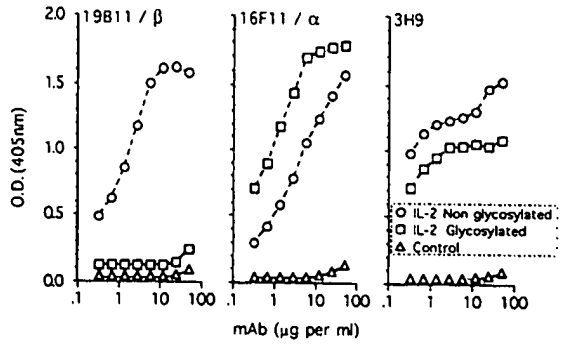

Fig. 8. Binding of mAbs to glycosylated or non-glycosylated rIL-2. The plates were coated either with glycosylated rIL-2 produced in CHO cells (Sanofi) or non-glycosylated IL-2 produced in *E. coli* (Roussel Uclaf). The binding was then analysed with the ELISA technique discussed in Materials and Methods.

MAb 16F11/α only blocks the binding to TS1α cells and not to TS1β, it does affect the binding of IL-2 to YT cells and only inhibits the IL-2 induced proliferation of TS1αβ cells. In addition, mAb 3H9 also used as control, further demonstrates the specificity of the effects observed with mAb 19B11/β.

The epitope recognized by mAb 19B11/β has been characterized using different peptides. It recognizes two peptides, namely 1–22 and 1–30 covering the NH_2 terminal part of IL-2 including the first 10 aa of the NH_2 terminal and either all (1–30) or about half of the α helix A (1–22). Its binding is totally specific and mAb 19B11/β does not recognize peptides 30–54 and 44–54. These data have been obtained by direct binding in ELISA and by inhibition experiments. The affinity of the two peptides (1–22 and 1–30) has been measured (K_d is between 10^{-7} M and 5×10^{-7} M). These data are in complete agreement with the observation that aa at positions 20 and 21 (Asp 20 and Leu 21) play an important role in IL-2/IL-2Rβ interaction.

The fine localization of the epitope recognized by mAb 19B11/β gives further insight into the interaction between IL-2 and IL-2β. Asp 20 and Leu 17 are not directly implicated in the 19B11/β epitope since various mutants of IL-2 with single aa substitution as well as double mutant at these positions are recognized as well as wild-type IL-2 by mAb 19B11/β (data not shown). The strong implication of threonine 3 was unexpected. This area of the molecule is not thought to have a well defined structure and is likely not to be the first site of interaction between IL-2 and IL-2β. The inhibitory effect of mAb 19B11/β on the interaction between IL-2 and IL-2Rβ may be due to either steric hindrance or to interaction with a secondary site of binding necessary for the stabilization of the IL-2/IL-2Rβ complex.

The observation that mAb 19B11/β does not recognize glycosylated IL-2 is in contradiction with the fact that it can, however, neutralize the biological activity of IL-2 produced *in vitro* by human peripheral lymphocytes stimulated by anti-CD3 mAb or phytohemaglutinin (data not shown). To explain this contradiction, one has to speculate that the epitope recognized by 19B11/β is localized in peptide 1–30, that threonine 3 plays a critical role in ELISA recognition but that in liquid phase other aa not hidden by the sugar moeity are accessible to the mAb and also play an important role in epitope structure.

The characterization of mAb 16F11/α and 3H9 have also provided some information. These mAbs recognize different epitopes and do not compete for the same site in IL-2 (Rebollo *et al.*, 1992). Unfortunately the epitope of mAb 16F11/α is conformational (Fig. 4) and therefore it was not possible to study this epitope related to the IL-2/IL-2Rα area of interaction. The epitope of mAb 3H9 was characterized. Its affinity for IL-2 and peptides 30–54 (A-B loop) and 44–54 is interesting to consider. Since mAb 3H9 has some affinity for peptide 44–54, one may speculate that part of its epitope is in this area even if additional aa from peptide 30–44 increases its affinity. This result can be compared to the data reported by Sauvé *et al.* (1991) showing the importance of Lys 35, Arg 38, Phe 42 and Lys 43 in IL-2/IL-2Rα interactions. MAb 3H9 does not inhibit significantly neither IL-2 binding to TS1α nor the IL-2 proliferation mediated by high-affinity IL-2 receptor (Fig. 3) maybe because of its relatively low affinity (Table 1). Furthermore, the observation showing that mAb 3H9 has greater affinity for peptide 30–54 than for IL-2 may indicate that the animals are immunized by a fragment of IL-2 produced after *in vivo* degradation or/and that in the IL-2 molecule this peptide has a conformation not easily accessible to the mAb 3H9.

Several observations made during the course of this work deserve some additional comments.

The high reactivity of polyclonal antibody from IL-2 immunized animals with peptide 1–30 shows that this area of the molecule is very immunogenic. This is independent of the glycosylation of the molecule since it has been shown that IL-2 produced in *E. coli* or in CHO cells are equally immunogenic (Sanofi, pers. comm.).

In Fig. 1 it has been shown that mAb 19B11/β stimulates the binding of radiolabelled IL-2 to TS1α cells. The work carried out in the mouse system shows that the loop including aa 30–54 as well as the α helix B-B′ plays an important role in the IL-2 binding to the α chain. One may speculate that the complex made by mAb 19B11/β and IL-2 is more stable and binds with more affinity to IL-2Rα. Alternatively, the avidity of IL-2 for IL-2Rα may be increased by the aggregation of two molecules of IL-2 on the surface of the IgG molecule. These effects also found with mAb 3H9 are dose-dependent and difficult to study.

The fact that mAb 3H9 binds peptides 1–22 and 1–30 in ELISA while it has no affinity for this peptide and the observation that on the contrary, it has significant affinity for peptide 44–54 while it does not recognize it in ELISA, further points to the necessity of measuring affinities before making any kind of conclusion on antibody–antigen interactions.

Mutagenic analysis of human IL-2 has already provided some information concerning the role of α helix A on the binding of this cytokine to the IL-2Rβ (receptor

of intermediate affinity in conjunction with the γ chain). The characterization of the epitope defined by mAb 19B11/β with specific inhibitory and functional properties described in the present paper provides complementary information. A detailed understanding of the biochemical mechanisms and structure–activity relationships of IL-2 and its receptor is required before it is possible to understand in more detail the fundamental properties of this cytokine and to develop new analogs for therapeutic applications.

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Advances in interleukin 2 receptor targeted treatment

John C Morris, Thomas A Waldmann

Abstract

T cell activation and cellular immune responses are modulated by interleukin 2 (IL2) through binding to its corresponding cell surface receptor. Three forms of the receptor are recognised based on IL2 binding affinity. The high affinity receptor is a heterotrimer composed of α, β, and γ_c-polypeptide chains. The 55 kDa α-chain also known as the Tac (T cell activation) antigen or CD-25 is a unique subunit of the high affinity IL2 receptor (IL2Rα). Resting T cells express few IL2Rα, however, when activated, the expression of ILR2α rapidly increases. The IL2Rα is shed from the cell surface and is measurable in the serum as a 45 kDa soluble form (s-Tac or s-IL2Rα). Serum concentrations of s-Tac can be used as a surrogate marker for T cell activation and IL2Rα expression. IL2Rα is over expressed by T cells in a number of autoimmune diseases, allograft rejection and a variety of lymphoid neoplasms. IL2 induced proliferation of T cells can be inhibited by the murine monoclonal antibody (anti-Tac) directed against the α-chain of the IL2R. Through molecular engineering, murine anti-Tac has been humanised reducing its immunogenicity without changing its specificity. Humanised anti-Tac (HAT) has been shown to reduce the incidence of renal and cardiac allograft rejection as well as decrease the severity of graft versus host disease in patients undergoing HLA matched allogeneic bone marrow transplantation. IL2Rα targeted treatment with radioimmunoconjugates of anti-Tac and immunotoxins has shown promise in the treatment of CD25 expressing lymphomas.

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In 1976, Morgan *et al* described an activity in the supernatants of cultured PHA stimulated peripheral blood mononuclear cells they termed T cell growth factor (TCGF) that was capable of stimulating the growth of bone marrow derived T lymphocytes.[1] Shortly thereafter, it was discovered that TCGF was required for the indefinite culture of cytolytic T cells in vitro.[2] In 1983, Taniguchi and coworkers using expression cloning isolated the cDNA for TCGF.[3] In recognition of its broad pleiotrophic activities, TCGF was subsequently renamed interleukin 2 (IL2).[4] It is a 153 aminoacid polypeptide with a 20 aminoacid signal sequence and a cysteine disulphide bridge. IL2 is a potent immunomodulator whose major function is the activation of various cells of the immune system including helper T cells, cytotoxic T cells, B cells, NK cells and macrophages.

IL2 mediates its biological effects through binding to its corresponding cell surface receptor. There are three recognised IL2 receptors (IL2R): a high affinity ($K_d = 10^{-11}$ M) receptor, an intermediate affinity ($K_d = 10^{-9}$ M) receptor and a low affinity ($K_d = 10^{-8}$ M) receptor.[5] These receptors are composed of up to three glycopeptide subunits: a 251 aminoacid 55 kDa α-chain (CD25),[6 7] a 75 kDa β-chain (CD122)[8] and a 64 kDa γ_c-chain (CD132).[9] The β- and γ_c-chains share significant homology with other members of the class I cytokine receptor family, however, the separate α-chains of the IL2R and the IL15R form a separate unique class. The high affinity receptor is a trimer consisting of an α-, β- and γ_c-chain.[10] The intermediate affinity receptor is a dimer composed of a β- and a γ_c-chain, and the low affinity receptor appears to be a monomeric α-subunit. The γ_c-subunit seems to be constitutively expressed on most lymphoid cells, but the expression of the α- and β-chains are more tightly regulated. The various polypeptide subunits perform different functions. The α-subunit mediates binding of IL2 and upregulates receptor sensitivity to IL2, the β-subunit also plays a part in binding of ligand, both the β- and γ_c-subunits mediate internalisation and signal transduction through the JAK 1 and 3 kinase pathways.[11 12] The IL2R and the IL15 receptor share a common β-chain, whereas the γ_c-subunit is shared among the IL2, IL4, IL7, IL9 and IL15 receptors.[13] Other surface molecules have been found to be associated with the IL2R including ICAM-1 (CD54) and MHC class I molecules.

Less than 5% of normal circulating peripheral blood mononuclear cells express the IL2Rα and then only at a very low level. The high affinity IL2R is not expressed on normal or unstimulated lymphocytes, but it is rapidly transcribed and expressed on activated T cells.[14 15] The 55 kDa α-polypeptide (IL2Rα) of the high affinity receptor, also known as the Tac (T cell activation) antigen, or CD25 is enzymatically cleaved and shed from the surface of expressing cells.[16] It can be measured in the serum by ELISA assay as a 45 kDa monomer called soluble-Tac (s-Tac) or soluble-IL2 receptor (s-IL2Rα).[17] The release of s-Tac is proportional to its cell surface expression.[18] It is excreted and catabolised by the kidneys and has a serum half life ($t_{1/2}$) of 0.62 hours. Serum values in normal healthy people range between 112–502 IU/ml (1 IU =

Metabolism Branch, Division of Clinical Sciences, National Cancer Institute, NIH, Bldg 10, Rm 4N115, 10 Center Drive, Bethesda, MD 20892–1374, USA

Correspondence to: Dr Morris (jmorris@mail.nih.gov)

3.3 picograms). The serum levels of s-Tac are increased in renal failure due to its decreased catabolism. Administration of exogenous IL2 will also increase serum s-Tac concentrations.[19]

Several murine monoclonal antibodies have been developed against the various subunits of the human IL2R and have been used for detection and diagnostic purposes as well as treatment. Included are anti-Tac (anti-CD25) that binds to an epitope on the α-chain and inhibits the binding of IL2 to its receptor,[20] 7G7/B6 that binds to a separate epitope on the IL2Rα and does not inhibit IL2 binding,[20] and Mikβ1 that binds to the IL2R β-chain (CD122).[21] Anti-Tac, but not Mikβ1 has been shown to inhibit IL2 induced proliferation of human T cells.[22] On the other hand, Mikβ1 inhibits IL2 and IL15 binding on large granular lymphocytes and the activation of these cells that expresses the IL2Rβ, but not the IL2Rα. MIKβ1 inhibits the action of IL15, but not IL2 on activated T cells that express the high affinity IL2 receptor. Anti-Tac and Mikβ1 act synergistically to inhibit IL2 induced proliferation in T cells.[21]

The therapeutic use of murine monoclonal antibodies has been limited because of their immunogenicity and the rapid development of neutralising human antimouse antibodies (HAMA) in patients receiving them.[24] Furthermore the short in vivo survival of murine antibodies limits their use in chronic diseases. Major efforts have gone into developing molecular techniques for "humanising" these antibodies to reduce their immunogenicity. In humanised anti-Tac about 90% of the molecule (murine IgG_{2a}) has been replaced with a human $IgG_{1\kappa}$ sequence.[25] Humanised anti-Tac (HAT) has the advantages of a low incidence of induction of neutralising antibodies, a significantly prolonged serum half life ($t_{1/2}$) of 20 days compared with 40 hours for the murine antibody, and the ability to mediate antibody dependent cytotoxicity (ADCC) through its humanised Fc-domain.[26]

Table 1 Causes of increased serum soluble IL2Rα (Tac) concentrations

Allograft rejection	*Neoplasia*
Bone marrow	Acute myelocytic leukaemia
Cardiac	Anaplastic large cell lymphoma
Liver	Adult T cell leukaemia/lymphoma
Renal	Chronic lymphocytic leukaemia
	Chronic myelocytic leukaemia
Autoimmune disease	Cutaneous T cell lymphoma
Aplastic anaemia	Mycosis fungoides
Behçet's syndrome	Hairy cell leukaemia
Crohn's disease	Hodgkin's disease
Giant cell arteritis	Non-Hodgkin's lymphomas (B cell)
Juvenile rheumatoid arthritis	Peripheral T cell lymphomas
Kawasaki disease	
Multiple sclerosis	*Veno-occlusive disease*
Polymalgia rheumatica	
Rheumatoid arthritis	
Sarcoidosis	
Scleroderma	
Sjögren's syndrome	
Systemic lupus erythmatosus	
Vasculitis	
Wegerner's granulomatosis	
Drugs	
In vivo IL2 administration	
End stage renal disease	
Infections	
HIV/AIDS	
Pulmonary tuberculosis	
Rubeola	
Infectious mononucleosis	
Sepsis	

IL2 receptor directed treatment in autoimmune disease

Little IL2Rα is expressed on unstimulated T cells or is found as s-Tac in the serum of healthy people, however, significant increases in IL2Rα are seen in the setting of autoimmmune diseases, organ transplant rejection and in many T cell and B cell neoplasms (table 1). There are a large number of reports evaluating the use of serum s-Tac levels as a measure of disease activity in autoimmune diseases.[27] Investigators have attempted to follow the levels of s-Tac as an indicator of systemic lupus erythematosus (SLE) disease activity with mixed results.[28 29] In one study, patients with SLE exhibited greater lymphocyte activation having increased circulating CD25+ cells compared with normal controls, but the increased expression did not correlate with exacerbations of their lupus.[11]

Similar findings have been reported in patients with rheumatoid arthritis (RA) where serum or synovial fluid s-Tac levels were found to correlate with disease activity or response to treatment in some studies but not others.[12 13] In a study by Suenaga and colleagues, two of three patients with increased s-Tac (> 2 SD above normal) and joint findings suspicious for but not diagnostic of RA, developed frank RA when followed up for one year compared with 25% of patients with joint pain and normal s-Tac levels.[30] Increased s-IL2Rα levels have been found to correlate with the systemic onset of juvenile rheumatoid arthritis (JRA) but not with onset of limited disease[17] and with activity of Sjögren's syndrome.[18] One report suggested that pleural effusions from RA could be differentiated from those of SLE based on the higher levels of s-Tac found in these effusions.[31] Soluble IL2Rα levels have been found to be increased in Wegerner's granulomatosis, vasculitis, polymyalgia rheumatica, giant cell arteritis, Kawasaki disease, and Behçet's syndrome

Experimental animal models of autoimmune uveitis[32] as well as uveitis patients demonstrate increased circulating T cells bearing large numbers of high affinity IL2 surface receptors. Ten patients with progressive chronic bilateral non-infectious sight threatening posterior and intermediate uveitis were weaned off of their systemic immunosuppression and treated with HAT 1mg/kg intraveneously at two week intervals for 12 weeks, then at three week intervals for 12 weeks and finally at four week intervals to complete one year of treatment.[33] Eight of the 10 patients had improvement of their visual acuity and two patients had progressive deterioration. Six patients developed transient rashes during the treatment as the only potential toxicity and no patient developed neutralising antibodies to the HAT. Additional benefits of the IL2R targeted treatment were that after the patients were tapered off of systemic corticosteroids, there were improvements in blood pressure and serum cholesterol levels.

IL2 receptor targeted treatment in organ transplantation

Serum concentrations of the soluble form of the IL2Rα has been found to be increased in patients rejecting allografts and activated T cells play a major part in graft rejection.[42] In the United States two monoclonal anti-IL2Rα antibodies have been clinically approved to prevent renal allograft rejection. Daclizumab (Zenapax, Hoffmann-LaRoche, Nutely, NJ) is the humanised monoclonal with approximately 90% of the murine sequence replaced by human sequences and basiliximab (Simulect, Novartis Pharma, East Hanover, NJ), a chimerical monoclonal antibody in which 75% of the sequence has been humanised.[43 44] The two antibodies are administered on different dose schedules because of their different pharmacokinetic profiles. Basiliximab is administered as a 20 mg intravenous dose two hours before transplant and again on the fourth postoperative day for a total of two doses. Daclizumab is approved to be administered intravenously at 1 mg/kg preoperatively and postoperatively at weeks 2, 4, 6, and 8 for a total of five doses. In a pilot study, 10 patients receiving living related renal allografts and two patients receiving cadaveric transplants were randomised to receive daclizumab 0.5 mg/kg or 1 mg/kg weekly, or 0.5 mg/kg or 1 mg/kg every two weeks beginning 12 hours before transplant for a total of five doses along with cyclosporine, corticosteroids and azathiaprine.[45] None of the allograft recipients experienced an episode of rejection in the first year, compared with 7 of 17 (41%) historical controls. In a US multicentre Phase III randomised double blind placebo controlled study, 260 patients undergoing first cadaveric renal transplant were randomised to standard treatment with cyclosporine, azathiaprine and corticosteroids, or this regimen plus daclizumab.[46] Of the 126 patient receiving daclizumab, 28 (22%) had a biopsy confirmed episode of rejection compared with 35% of 134 control patients receiving standard immunosuppression plus placebo (p = 0.03). There was, however, no statistically significant difference in graft survival between the two treatment groups at one year (95% *v* 90%, p = 0.08) and there was no difference with respect to adverse drug reactions, infectious complications or the incidence of cancer between the groups. In a second European multicentre Phase III randomised double blind placebo controlled study daclizumab was compared with placebo in 275 patients receiving a less intensive immunosuppressive regimen of cyclosporine and corticosteroids receiving their first cadaveric renal transplant.[47] At six months 28% of patients on the daclizumab arm had a biopsy confirmed episode of acute rejection compared with 47% of patients in the placebo arm (p = 0.001). Patients on the daclizumab treatment arm had better graft function, reduced need for antithymocyte or antilymphocyte globulin, lower administered corticosteroid doses, a lower incidence of cytomegalovirus infections, a lower incidence of infectious deaths and a greater one year survival than patients on the placebo arm (99% *v* 94%, p = 0.01). Randomised controlled clinical trials using basiliximab in renal transplantation have shown similar encouraging results.[44]

IL2Rα receptor directed treatment has also been shown to improve the survival of other organ allografts. In a primate cardiac allograft model, the efficacy of HAT was compared with murine anti-Tac for the prevention of rejection.[48] Both monoclonal antibodies resulted in superior allograft survival compared with controls, however, HAT was less immunogenic and resulted in a better survival compared with the murine antibody. Recently, 55 patients undergoing first cardiac allograft were randomised to cyclosporine, mycophenolate mofetil and prednisone with or without daclizumab 1 mg/kg intravenously administered every two weeks for five doses.[49] Acute rejection occurred in 17 of 27 patients on standard immunosuppression and in 5 of 28 patients on the standard immunosuppression plus HAT treatment arm (p = 0.04). In addition, the severity of the rejection episodes was reduced and the time to first episode of rejection was prolonged in the HAT treatment group. No increased toxicity or higher incidence of infection or cancer was seen in the HAT treatment arm.

Mixed results have been seen in trials using HAT for patients undergoing liver transplantation. In one study 28 patients undergoing a liver transplant were treated with prednisone, cyclosporine and HAT and no episodes of acute rejection were reported.[50] Recently, a pilot study was terminated after seven of seven patients developed rejection when treated with a regimen of corticosteroids, mycophenilate moefitel and daclizumab without cyclosporine or other calcineurin inhibitor compared with 36% of patients treated with corticosteroids, mycophenolate mofetil and a calcineurin inhibitor.[51] These investigators suggested the high rejection rate was the result of the absence of a calcineurin inhibitor in the immunosuppressive regimen and the lower than anticipated serum levels of daclizumab in hepatic transplant patients compared with renal transplant patients.

IL2 receptor targeted treatment is being evaluated for the prevention and treatment of graft versus host disease (GvHD). GvHD is the most frequent complication of allogeneic bone marrow transplantation occurring in 30–60% of HLA matched sibling transplants.[52 53] The incidence of GvHD is as high as 90% in matched unrelated donor transplants and the mortality of acute grade 4 GvHD approaches 100%.[54] GvHD is mediated by the reactivity of mature T cells in the donor marrow against recipient alloantigens.[55–57] Donor T cells stimulated by alloantigens secrete IL2 and express IL2Rα.[58] Several studies have shown an association of soluble IL2Rα levels and the activity of GvHD.[59–61] Serum soluble Tac levels increase at the time of engraftment and at the onset of acute and chronic GvHD.[61] The peak concentration of s-ILRα correlated with the severity of the GvHD and falls as the patients' GvHD resolves.[62 63] Prolonged increase in serum s-IL2Rα is often followed by the development

of chronic GvHD. Patients with large increases of their s-IL2Rα levels have a poorer survival compared with patients with mild increases. In contrast, other studies have found little predictive or diagnostic role for monitor of s-Tac as an indicator of GvHD. Sepsis, veno-occlusive disease and other conditions common in the bone marrow transplant setting other than GvHD can cause increase in the serum s-IL2Rα levels.[64]

GvHD is treated with corticosteroids and immunosuppressive agents, however, the use of anti-CD25 antibodies is receiving increasing attention as an adjunct to treatment or for patients who are resistant to corticosteroids. Prophylactic use of monoclonal antibodies to IL2Rα reduces the incidence and severity of GvHD in murine models.[65] Early clinical trials, however, were disappointing. A study using a rat monoclonal antibody (LO-Tact-1) directed against IL2Rα as prophylaxis for GvHD in 10 patients undergoing HLA matched sibling donor bone marrow transplant found it had no benefit in reducing the incidence of GvHD, the frequency of relapse or overall survival.[66] Studies using an IL2-*Pseudomonas* exotoxin A fusion protein or a *Pseudomonas* exotoxin A-anti-Tac Fv fragment have been shown to reduce circulating CD25+ T lymphocytes, reduce allorcactive T cells by 100-fold and decrease the incidence of GvHD in mice transplanted with allogeneic cells.[67]

Two recent studies suggest a benefit of humanised anti-Tac (daclizumab) for the treatment of GvHD. In a study of 20 patients with corticosteroid refractory acute GvHD who were treated with doses of daclizumab ranging from 0.5 mg/kg to 1.5 mg/kg, four complete and two partial responses were seen.[68] Toxicity was limited to chills and diaphoresis in one patient and no patient developed an antibody response to the humanised antibody. In a second study, 24 patients with advanced or corticosteroid refractory GvHD were treated with 1 mg/kg daclizumab on days 1, 8, 15, 22, 29.[69] Patients were evaluated for response of their GvHD on day 43. The complete response rate for this group of patients was 29% and the survival on day 120 was 29%. In the same study, a second cohort of 19 patients received daclizumab 1 mg/kg on a more intensive schedule (days 1, 4, 8, 15 and 22). The complete response rate on day 43 was 47% and survival on day 120 was 53%. No significant toxicity was seen in either cohort, but a reduction of serum s-IL2Rα and circulating CD3+, CD25+ cells were noted. These laboratory changes were not predictive of outcome.

IL2 receptor targeted treatment of neoplasia

The greatest expression of IL2Rα and highest s-Tac levels are seen in the setting of T cell malignancies. This coupled with the ability of anti-Tac to block the binding of a specific cytokine growth factor (IL2) to its receptor, inhibiting proliferation of the malignant cell, make this an ideal target for specific monoclonal antibody treatment. Over expression of IL2Rα is seen on the cells of adult T cell leukaemia/lymphoma (ATL), mycosis fungoides, peripheral T cell lymphomas, hairy cell leukaemia, Reed-Sternberg cells (Hodgkin's disease), anaplastic large cell lymphoma and some B cell neoplasms.[70]

ATL has served as the prototypical disease for the therapeutic trials of anti-Tac. ATL is an aggressive lymphoproliferative disorder caused by infection with a novel human retrovirus: the human T cell lymphotrophic virus type 1 (HTLV-1).[71 72] HTLV-1 infection is geographally clustered found primarily in the Caribbean basin, Western Africa and Southern Japan.[73] After infection, reverse transcription and integration of the virus into a host T cell genome, expression of the virally encoded 42 kD *tax* protein acts as a promiscuous transcriptional activator binding to the promoters of multiple families of genes, most notably the IL2, IL2Rα and NFκB genes.[74] This results in increased gene expression and dysregulation of cell growth. About 5% of infected people will develop an aggressive form of T cell leukaemia or lymphoma[75] characterised by a CD3+, CD4+, CD25+ phenotype.[76] In its most aggressive form, ATL patients developed high circulating cell counts, severe hypercalcaemia, lytic bone lesions, skin and solid organ infiltration, liver failure, leukaemic leptomeningitis and profound immunosuppression. The median survival for patients with acute ATL is approximately five months.[77] Chemotherapy has had little impact on the disease with relatively few patient responses and a short duration of remission. Even the combination of interferon alfa and zidovudine (AZT), a treatment used throughout much of the world results in a median survival of only three months.[78 79]

Beginning in the 1980s, a series of clinical trials were initiated in the Metabolism Branch of the NCI by Waldmann and colleagues using monoclonal anti-Tac antibodies as a therapeutic approach to ATL. In the first trial, 19 patients with acute ATL were treated with unmodified murine anti-Tac.[80] Ten of the 18 patients had failed prior chemotherapy. Two patients achieved complete remissions, four had a partial response and one patient a mixed response. The duration of remissions ranged from nine weeks to more than three years. Toxicities included fever in two patients and transient pancytopenia in one patient. The short serum half life of murine anti-Tac and the development of human antimouse antibodies (HAMA) limited the usefulness of this approach. In a follow up phase I/II study, murine anti-Tac was armed with the β emitting isotope ^{90}Yttrium in an effort to enhance leukaemic cell killing.[81] Eighteen patients with ATL were treated with 5–15 mCi ^{90}Y labelled murine anti-Tac. Two complete and seven partial responses were seen in 16 evaluable patients. Toxicity was confined to the haematopoietic system, primarily granulocytopenia and thrombocytopenia. However, a significant number of patients developed HAMA titres limiting the ability to administer repeated doses of the antibody. With the approval and availability of

humanised anti-Tac (dacluzimab), the Metabolism Branch has pursued clinical trials using ^{90}Y labelled HAT in ATL, other CD25 expressing T cell neoplasms and Hodgkin's disease. Recently we initiated a phase I/II trial of high dose (4–8 mg/kg) unmodified HAT as a treatment for patients with of ATL. End points for this study are dose limiting toxicity, the ability to achieve > 95% anti-Tac saturation of the IL2Rα on circulating ATL cells or in lymphomatous tissue (lymph nodes, skin, etc) and tumour response. Future approaches under development are the use of HAT chelated to α emitting radionuclides such as ^{212}Bi, ^{211} Bi or ^{211}At because of their more favourable radiobiological profiles, as well as the use of non-cross reactive antibodies such as high dose unmodified HAT in combination with low dose radioisotope armed humanised 7G7/B6 to block IL2 stimulation (HAT) and deliver a lethal dose of radioactivity to the malignant cells (radiolablled 7G7/B6). Combination of anti-IL2R antibodies with other antibodies (anti-CD30) or with other modalities such as chemotherapy or other cytokines is also projected.

Other approaches to IL2R targeted treatment of cancer have been the use of ligand-toxin fusion proteins and immunotoxins. $DAB_{389}IL\text{-}2$ is a 58 kDa fusion protein engineered between the enzymatic and translocation domains of diphtheria toxin (DT) and human IL2 that can be expressed in *Escherchia coli*.[83] This fusion protein is able to direct cytocidal activity to cells that express the IL2 receptor. The toxin is unable to be taken up by other cells because it lacks the cell receptor binding domain. When bound to cells expressing the IL2R and internalised, the DT kills cells by catalysing the irreversible ADP-ribosylation of elongation factor-2 (EF-2) and subsequent inhibition of protein synthesis. It has been estimated that even one cytosolic molecule of DT is lethal to the cell.[84] In a clinical trial involving 35 patients with advanced treatment refractory cutaneous T cell lymphoma and mycosis fungoides, DAB_{389} IL2 produced a 37% response rate including 14% complete responses.[84] Toxicities included fever/chills, hypotension, nausea/vomiting and liver enzyme abnormalities. A recent trial using a fusion protein of a truncated *Pseudomonas* exotoxin A and the Fv-fragment of anti-Tac (LMB-2) showed promising early results in patients with I-2Rα expressing lymphoid neoplasms including hairy cell leukaemia and ATL.[85]

The field of receptor targeted treatment is still in its infancy. No doubt with continued improvement of monoclonal antibody technology, refinments in linking toxins and radiopharmaceuticals to antibodies and ligands, the targeting of the IL2R and other cytokine receptors holds great promise as treatment for a large number of diverse diseases.

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